Phytase From Transgenic Alfalfa for Supplementation of Poultry and Swine Rations

R.G. Koegel, S. Austin-Phillips, M.E. Cook and R.J. Straub

Introduction

Buildup of phosphorus in the environment and the resulting degradation of water resources is of mounting concern. Much of this buildup is traceable to human activities. Important among these is livestock production. Monogastric animals, such as poultry and swine, which can solubilize only a small fraction of the phosphorus in their grain-based rations while excreting the remainder, have come under increased scrutiny. Supplementation of inorganic phosphorus into rations to meet animal nutritional requirements exacerbates the problem.

Much of the phosphorus in grain is in the form of insoluble phytates. Researchers have shown that supplementing poultry and swine rations with the enzyme phytase can lead to solubilization of the phosphorus, thus eliminating the need for phosphorus supplementation and concurrently reducing the level of phosphorus in the excrement to approximately one-half of that normally experienced.

Because of relatively higher population and animal densities in western Europe, concern over phosphorus buildup has preceded that in the U.S. Accordingly, certain areas, like the Netherlands, have mandated limits on animal numbers and/or required the use of phytase in animal rations.

The enzyme phytase derived from *Aspergillus niger* has, to date, generally been produced in fermentation vats using genetically engineered microorganisms. It has been estimated that the cost of phytase supplementation with this material would be about three times the cost of conventional supplementation with dicalcium phosphate.

As an approach to reducing the cost of phytase production, a multi-disciplinary ARS-UW team at Madison, Wisconsin has produced transgenic alfalfa with the capability of expressing phytase. This phytase can be recovered with juice extracted from the herbage. Other constituents of the juice including xanthophyll, used to pigment egg yolks and broiler skin; high levels of dietary protein and various vitamins and minerals add to its value in rations. The use of whole alfalfa herbage, however, would not be desirable due to its high fiber content. Since phytase would potentially be needed in great quantities, but not in very pure or concentrated form, it is believed that the economic advantage of production in “plant bioreactors” such as alfalfa would be great. The advantage in capital costs is particularly great. Ideally, the cost of phytase supplementation should be competitive with the traditional dicalcium phosphate supplement, with the environmental benefits as an added incentive.

Methods

Through collaborative efforts with Dr. David Russell (Agracetus), we obtained *Agrobacterium tumifaciens* strains containing a derivative of the pBI binary vector in which the *Aspergillus niger* phytase gene had been placed under the control of either the CaMV 35S promoter or the *Arabidopsis thaliana* Rubisco small subunit (SSU) promoter. Both constructs also incorporated a signal peptide for targeting of the phytase enzyme to the apoplast. We also constructed an additional expression cassette utilizing the hybrid “MAC” promoter, which contains elements of both the CaMV 35S and *Agrobacterium* nopaline synthase promoters. This promoter was fused to the *A. niger* phytase gene (provided by Dr. Edward J. Mullaney, USDA), again incorporating a signal peptide for apoplast localization. This expression cassette was cloned into a pCGN binary vector and mobilized into *Agrobacterium*.

Transgenic plants were propagated by cuttings to generate a sufficient number of plants for juice expression as described below. Whole juice was used in a preliminary feeding trial in which day old chicks were fed a grain-based diet amended with either dicalcium phosphate (at levels corresponding to 0, 33, 66, or 100% of NRC requirement) or alfalfa juice.

Results

In vitro transformation of both tobacco (W38) and alfalfa (RSY27) was accomplished with all three expression constructs. Significant levels of protein expression were obtained in all cases. Although plant-expressed phytase was underglycosylated in both tobacco and alfalfa (based on mobility on SDS-PAGE), the enzyme retained stability to high temperature (55 °C) and low pH (2.5). Comprehensive analysis of transgenic phytase-expressing plants is currently underway.

Of the transgenic alfalfa plants obtained, those transformed with the 35S promoter construct gave the highest levels of phytase expression, with several individual transgenic plants yielding phytase activity corresponding to 1 - 2% of total extracted protein.

During the 3 week trial (Table 1), chicks fed control diets lacking calcium phosphate either with or without control juice (expressed from non-transgenic RSY27) did not survive. While those chicks receiving phytase-amended feed (formulated at 400 units phytase per Kg feed) did not do as well as those receiving calcium phosphate, their relatively good performance indicated that the use of phytase-containing alfalfa juice is a very practical alternative to the addition of inorganic supplemental phosphate to the diet. It should be noted that the chicks with alfalfa juice in their diet received less calcium than those fed monocalcium phosphate. Furthermore, it is not known whether phytase was fed at the optimum level.

Plant-expressed phytase retained the stability observed for the *A. niger* enzyme. When either lyophilized or whole phytase-containing alfalfa juice was added to poultry feed at 400 units/Kg, no appreciable loss of activity was observed over 3 weeks of storage at room temperature (22 °C). In addition, we have verified that phytase in alfalfa juice (at 400 units/Kg) can efficiently release phosphate from phytase present in poultry feed in vitro.

Conclusions

Phytase was produced in transgenic alfalfa at concentrations of over 1½% of the soluble protein. Juice from this alfalfa proved to be effective in replacing inorganic phosphorus supplements in the diets of chicks.

Table 1. Chick feeding trial comparing inorganic phosphorus supplementation with phytase from alfalfa juice — corn/soybean diet. 25 chicks per treatment. November 1996.

Treatment	Week 1		Week2*		Week 3*	
Monocalcium phosphate						
(% P in diet)	Gain	Feed/gain	Gain	Feed/gain	Gain	Feed/gain
0%	**					
0.05%	83	1.167	245	1.365	504	1.495
0.10%	81	1.340	268	1.244	604	1.345
0.21%	87	1.345	304	1.357	597	1.515
Alfalfa juice						
No phytase	**					
400 IU Phytase/kg feed	86	1.336	244	1.560	492	1.431

*Numbers are cumulative from beginning of trial.

**Chicks euthanized after week 1 due to declining condition.

Research will continue in four areas:

1. Propagation of high-producing transformants to yield enough herbage for additional feeding trials.
2. Plant breeding to produce cultivars with good phytase yield, persistence, and high production characteristics.
3. Evaluation of alternative processes to transform juice into stable form(s) acceptable to the feed industry.
4. Additional feeding trials with both poultry and swine to determine optimum levels of phytase, stability over time, and reduction of phosphorus in feces.